

kinases known as Pyk2 and p125FAK (FAK). The Applicants have shown that HBx activation of Pyk2, FAK, Src and MAPK signalling, all occur in a calcium-dependent manner in that treatment of cells with calcium chelator (EGTA) or calcium channel poison (bis-(o-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid or BAPTA-AM) specifically blocks HBx stimulation of Pyk2, which is essential for HBx activity. In addition, treatment of cells with cyclosporin A (CsA), a specific inhibitor of mitochondrial voltage-dependent anion channels, which deregulates calcium channels, also impairs HBx stimulation of HBV genomic DNA replication. Thus, the Applicants have demonstrated that HBx functions through a calcium-dependent pathway to stimulate viral DNA replication in cells and Pyk2 signal transduction, which plays a fundamental role in mammalian hepadnavirus replication.

On pages 14-15, please replace the description of Figure 8 with the following:

FIGURE 8. Cells were propagated as described (Klein, et al. 1997, EMBOJ 18: 5019-5027), transfected with 5 µg of pΔBS empty plasmid (vector) or pAdCMVX (HBx) expression plasmid (Klein et al., 1997, Mol. Cell. Biol. 17: 6427-6436; Klein et al., 1999, EMBOJ 18: 5019-5027; Doria et al., 1995, EMBOJ 14: 4747-4757), 2 µg of luciferase reporter plasmid controlled by a minimal TATA-box promoter and 4 copies of an AP-1 binding site (Klein et al., 1997, Mol. Cell. Biol. 17: 6427-6436), and 5 µg of PKM plasmid expressing a dominant-interfering form of Pyk2 or pRK5 empty plasmid (Dikic et al., 1996, Nature 383: 547-550). Cells were allowed to recover for 12 h following transfection, then serum starved for 16 h. Chang cells are a human transformed hepatoblastoma line, HepG2 cells are a human differentiated hepatocytic line, and GN4 cells are a rat liver epithelial line. (A) Equal protein

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amounts were assayed for luciferase activity. For analysis of low level expression of HBx, 0.4 μ g of pAdCMVX was transfected. A typical experiment is shown. (B) Cell lysates were prepared in a modified RIPA buffer (Schlaepfer et al., 1998, Mol. Cell. Biol. 18: 2571-2585), gel-electrophoresis and immunoblot analysis was performed with anti-Pyk2 or anti-Y(P)-402 Pyk2 antibodies (Biosource, Int.). Nontransfected cells were treated with 20 ng/ml TPA for 20 min to activate Pyk2 (TPA samples). (C) Equal amounts of protein lysates were immunoprecipitated with antibodies to Pyk2, an in vitro kinase assay was performed using [γ - 32 P]ATP as described (Klein, et al. 1997, EMBOJ 18: 5019-5027), and phosphorylation of associated Src, Fyn, and Pyk2 analyzed by gel-electrophoresis and autoradiography. Identification of Pyk2 and Src-Fyn proteins, which electrophoretically comigrate, was performed by immunoblot with specific antisera (not shown). (D) Fyn was immunoprecipitated and autophosphorylation activity (Fyn assay) determined by in vitro kinase assay using [γ - 32 P]ATP, gel-electrophoresis and autoradiography as described (Klein et al., 1997, Mol. Cell. Biol. 17: 6427-6436; Klein, et al. 1997, EMBOJ 18: 5019-5027). Total Fyn protein level was determined by immunoblot of an equal fraction of the immunoprecipitate.

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On page 27, please replace the last paragraph with the following:

In particular, compounds which may be used in accordance with the present invention to specifically target mitochondrial calcium channels and regulatory components thereof. Compounds which may also be used in accordance with the present invention include those which specifically target endoplasmic reticulum calcium channels, SERCA Ca²⁺ pumps and regulatory components thereof. Compounds which may be used in accordance with the present invention include: Cyclosporin A, Dihydropyridines: nifedipine (Procardia),

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nimodipine (Nimotop), amlodipine (Norvasc), felodipine (Plendil and Renedil), isradipine (DynaCirc), nicardipine (Cardene), nisoldipine; Benzothiazepine: diltiazem (Cardizem), Phenylalkylamine, verapamil (Calan and Isoptin), Diarylaminopropylamine ethers, bepridil; Benzimidazole-substituted tetralines, mibefradil Piperazine, flunarizine (Sibelium); (+)-verapamil hydrochloride, omega-Agatoxin TK, omega-Agatoxin Iva, amiloride, Hydrochloride, nimodipine; (+)-Methoxyverapamil, ω-Agatoxin IVA, aminohexahydrofluorene, bepridil, calcicludine, calciseptine, diltiazem, flunarizine, FS2, galanin, HA 1004, HA 1077, nifedipine, nimodipine, nitrendipine, TaiCatoxin, protopine; cyclosporin A; 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate (BAPTA), BAPTA-AM, MAPTAM, EGTA.

On page 46, please replace the first full paragraph with the following:

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Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the desired modulating effects, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from in vitro data; e.g., the concentration necessary to achieve a 50-90% inhibition of HBV infection using the assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays, bioassays or immunoassays can be used to determine plasma concentrations.

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On page 58, please replace the last full paragraph with the following:

Ras-specific monoclonal antibody Y13-259. (Santa Cruz Biochemicals, Inc.). Rabbit anti-Shc and anti-Sos serum. (Santa Cruz Biochemicals, Inc.). Rabbit anti-Grb2, anti-ERK2, anti-Csk, anti-JNK and anti-N-Myc were purchased from Santa Cruz. Anti-Src (M327) antibodies were purchased

85 from Oncogene Science. Anti-Fyn antibodies (Santa Cruz) were a gift of D. Littman (NYU). Anti-phosphotyrosine (4G10; UBI) and rabbit anti-p34cdc2 serum and Rabbit anti-RPTP α serum. (Upstate Biotech Inc.).

On pages 80-81, please replace the paragraph spanning pages 80-81 and the following paragraphs with the following:

86 Studies determined whether HBx acts on intracellular calcium to activate Pyk2. HBx transfected Chang cells showed 5 fold increased phosphorylation of Pyk2 at Y-402, similar to TPA stimulation (Figure 9A). Treatment with the cell permeable cytosolic calcium chelator BAPTA-AM at 50 μ M (2 times IC₅₀) for 2 h prevented Pyk2 phosphorylation without altering Pyk2 levels (Figure 9A). HBx activation of Pyk2 therefore involves cytosolic calcium action. Studies next determined whether HBx acts on calcium channels in the endoplasmic reticulum (ER), mitochondria or plasma membrane (PM) for its activity. A low (0.5 mM) concentration of EGTA was added to the culture medium for 2 h to block entrance of extracellular calcium (Zwick et al. 1999, J.B.C. 274: 20989-20996), or cells were treated with BAPTA-AM to block ER and mitochondrial calcium, or cyclosporin A (CsA) to block mitochondrial calcium function. EGTA had no effect whereas BAPTA-AM or CsA both prevented HBx activation of Pyk2, indicating that HBx acts on ER/mitochondrial calcium control. A high concentration of EGTA (3 mM) did not block TPA activation of Pyk2 phosphorylation (Zwick et al. 1999, J.B.C. 274: 20989-20996) (Figure 9C), but partially inhibited activation by HBx. Therefore, HBx acts on the control of ER/mitochondrial calcium, with low level entry of extracellular calcium, suggestive of constitutive cytosolic calcium alteration (Clapham 1997, Cell 80:259-268).

The requirement for cytosolic calcium and HBx activation of Pyk2 in HBV replication was examined. HepG2 cells were

transfected with a 130% head-to-tail DNA copy of the HBV genome which replicates authentically in the livers of transgenic mice (Guidotti et al. 1995, J. Virol. 69: 6158-6169), in *Tupaia* hepatocytes in culture (Melegari et al., 1998, J. Virol. 72: 1737-1743), and in an HBx-dependent manner in HepG2 cells (Melegari et al., 1998, J. Virol. 72: 1737-1743). Expression of HBx was abolished by a targeted frameshift mutation (Melegari et al., 1998, J. Virol. 72: 1737-1743). HepG2 cells were transfected with vector alone, wild type HBV genomic DNA, or HBx(-) genomic DNA, cytoplasmic viral core particles, the structures in which viral DNA replication takes place, were isolated and the level of viral DNA replication was examined (Figure 9D). HBV DNA replication was reduced 20 fold in the absence of HBx expression, but recovered by cotransfection of HBx. Northern mRNA analysis demonstrated no reduction in HBV pregenomic (pg)RNA and HBsAg mRNAs in the absence of HBx (Figure 9D). Cotransfection of wild type HBV genomic DNA with PKM reduced viral DNA replication by 15 fold, similar to HBx(-) HBV samples, without altering viral mRNA levels (Figure 9E). These results demonstrate that HBx specifically promotes HBV DNA replication in a Pyk2-dependent manner.

On pages 82-83, please replace the paragraph spanning pages 82-83 with the following:

The requirement for cytosolic calcium in HBx-dependent viral replication was investigated. Cells transfected with wild type or HBx(-) HBV genomic DNA were treated for 4 d with 1 or 3 μ g/ml of CsA to block mitochondrial calcium channels. There was no evidence for CsA toxicity during treatment. CsA reduced HBV DNA replication in cytoplasmic core particles by 15 fold compared to untreated controls (Figure 10A), similar in magnitude to inhibition of Pyk2 or the absence of HBx expression. Northern mRNA analysis demonstrated a 2 fold

reduction in pgRLNA and HBsAg mRNAs (Figure 10A). To determine whether inhibiting cytosolic calcium and Pyk2 activity inhibits HBV DNA replication, HepG2 cells were transfected with HBV genomic DNA and treated with CsA, or cotransfected with PKM. Cytosolic core particles were purified and incubated with [α - 32 P]-dNTPs to examine endogenous HBV polymerase activity (Figure 10B). In untreated controls, predominantly full-length double-strand DNA products were produced, indicative of pgRNA reverse transcription and DNA-dependent DNA synthesis. PKM inhibition of Pyk2 or treatment of cells with CsA prevented DNA replication by 7 and 12 fold respectively. Treatment of HBV genome transfected cells with low levels of BAPTA-AM for 4 d impaired viral DNA replication by 10 fold without strongly reducing HBV mRNA levels (Figure 10C). Collectively, these data show that HBx activation of HBV reverse transcription and DNA replication involves alteration of cytosolic calcium and coupled activation of Pyk2. The requirement for cytosolic calcium in HBx transcriptional stimulation was investigated in HepG2 cells transfected with luciferase reporters controlled by transcription factor AP-1 or CREB, with or without treatment of cells by CsA (Figure 10D). HBx activation of AP-1 dependent transcription was impaired 2.5 fold by treatment of cells with 10 μ g/ml CsA. HBx stimulation of CREB-dependent transcription was resistant to high dose CsA treatment, consistent with HBx activation of CREB by direct interaction (Andrisani et al., 1999, J. Oncol. 15: 1-7). These data indicate that HBx transcriptional activation of AP-1 but not CREB requires alteration of cytosolic calcium.